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Evaluation of serum neopterin levels in patients with Graves disease

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ABSTRACT

Aims: Graves' disease is a disease with an autoimmune basis in which the synthesis and release of thyroid hormone from the thyroid gland increases. Interferon-gamma (IFN-γ) released from activated T lymphocytes causes macrophages to produce neopterin (NPT), increasing its concentration in serum and other body fluids. There is a relationship between NPT and the production of free oxygen radicals by these cells. In this study, it was aimed to measure serum NPT levels in individuals with Graves' disease.

Methods: The study included 13 newly diagnosed Graves' patients (neopterin levels were measured at the time of first diagnosis and at the 3rd month of treatment) and 16 Graves' patients who were followed up in endocrinology outpatient clinics for at least one year. NPT levels of 23 healthy individuals without any disease were taken as the control group. Free triiodothyronine (T3), free thyroxine (T4), thyroglobulin, and thyroid stimulating hormone (TSH) levels were measured in the blood samples of the participants.

Results: Serum NPT levels were found to be higher in Graves' patients compared to the control group (6.66 nmol/L in newly diagnosed patients, 9.24 nmol/L in patients at the 3rd month of treatment, 10.68 nmol/L in patients followed for one year or more, 1.44 nmol/L in the control group, respectively, $p < 0.05$).

Conclusion: Monitoring NPT levels in serum and other body fluids can be used to evaluate the diagnosis, prognosis, and treatment efficacy of autoimmune diseases. In addition, NPT can be suggested as a potential biomarker to determine disease stage, treatment efficacy, and immunological remission status, as well as the diagnosis of Graves' disease.

Keywords: Neopterin, Graves disease, hyperthyroidism, cellular immunity, T helper cells

INTRODUCTION

Graves disease is an autoimmune disease of the thyroid gland that can lead to hyperthyroidism. It was pointed out that Graves disease affects 1-1.5% of the population, and also, 4 out of 5 cases of hyperthyroidism are due to Graves disease. Graves disease is more common in women and adults older than 30.[1-3]

Infiltration of thyroid-antigen-specific T-cells into the thyroid gland and development of autoantibodies (TSH-R-Ab) against thyroid-stimulating hormone receptor (TSH-R) are involved in the pathogenesis of Graves disease. Activation of thyroid-stimulating hormone receptors by autoantibodies enhances intracellular cyclic AMP production, mediating thyroid hyperplasia and inappropriate thyroid hormone secretion.[4-6]

Neopterin (NPT), a pteridine, is an oxidized metabolite of tetrahydrobiopterin (BH_4) produced by activated macrophages. There is growing interest in considering NPT as a biomarker in infectious and inflammatory diseases and malignancies. The innate immune system activation leads to the stimulation of T-helper 1 cells, which secrete interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) and regulate tissue damage by the cellular immune response. During the inflammatory conditions, NPT is generated from BH_4, which has antioxidant effects with GTP cyclohydroxylase enzyme in macrophages induced with IFN-γ secreted from T-helper 1 cells. It can be put forward to NPT is considered a convenient biomarker to monitor immune activation in inflammatory conditions because the produced amount of NPT is closely related to immune system activation, and it can be easily measured in biological fluids.[7,8]

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Although it was a different study, our aim in this study was to compare Graves patients with high disease activity at different stages with a healthy control group with biochemical and hematological parameters.[9]

METHODS

The study was carried out with the permission of Eskisehir Osmangazi University Non-interventional Clinical Researches Ethics Committee (Date: 03.11.2020, Decision No: 2020-10). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

The study was conducted with 29 patients with Graves disease and 23 healthy volunteers. Patients over 18 years of age and without kidney failure or kidney damage were included in the study. Patients with autoimmune diseases other than Graves disease that would affect serum NPT levels, active acute or chronic viral/bacterial infection, and malignancy were excluded from the study. Graves disease was determined with undetectable TSH levels, elevated serum free T4 and free T3 concentrations, positive TSH receptor antibodies, and goiter and increased blood flow on thyroid ultrasound.[10]

According to the disease's duration, Graves disease patients were classified as 16 newly diagnosed and 13 as follow-up patients. The volunteer participants of the study were chosen from patients admitted to the Endocrinology outpatient clinic of Eskişehir Osmangazi University Hospital between December 2020-April 2021.

We evaluated newly diagnosed patients two times, at the first diagnosis and in the 3rd month of the treatment, to investigate the immunological response of these patients to anti-thyroid therapy. The other 13 patients with Graves (FUPG group) were previously diagnosed and were not in remission yet. According to the recommendations in the 2018 European Thyroid Association Guidelines, considering the severity of the disease and adjusting the dose, methimazole treatment was started, and methimazole treatment was continued in the FUPG, in accordance with the guideline recommendation.[1]

Blood samples collected from the participants were centrifuged at 3500 rpm for 10 minutes and stored at-80°C for measurements.

Serum neopterin concentrations were analyzed with commercial enzyme-linked immunosorbent assay (ELISA) kits (Human Neopterin Assay Kit, Bioassay Technology Laboratory, China) according to the manufacturer's instructions. Optical density was measured at 450 nm with a microplate reader (Spectra Max M2, England). Serum neopterin levels were expressed as nmol/L.

In all cases, free thyroxine (T4), free triiodothyronine (T3), thyrotropin (TSH), anti-TG, and anti-TPO were determined by Electro Chemiluminescent immunoassay and serum C-reactive protein (CRP) levels were measured by immunoturbidimetric method (Cobas e801 Roche Diagnostics, Mannheim, Germany) commercially available kits (T4, T3, TSH, anti-TPO, CBC analyses were performed on Sysmex XN 1000 hematology analyzers (Sysmex Corporation, Kobe Japan). Erythrocyte sedimentation rate (ESR) was studied by the Westergren method in a fully automated Vacuplus ESR-120 (Ankara, Turkey) analyzer.

Data were presented as mean±standard error mean. The conformity of the data to the normal distribution was evaluated with Shapiro-Wilks or Kolmogorov-Smirnov test. Data were statistically analyzed using the Kruskal-Wallis Test post hoc Bonferroni. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) 21. If $p<0.05$, the difference between the means was considered significant.

RESULTS

The patients with Graves disease included in this study were classified as newly diagnosed Graves patients (NDG) and followed-up Graves patients (FUPG). The N numbers of the participants and the mean values of their ages are given in Table 1. There was no significant difference in terms of age, gender, and BMIs between the groups. The followed-up Graves patients (FUPG) had 2.6 mean disease years from diagnosis.

Table 1. Demographic data of the participants. Data were presented as mean±SEM

		Control	NDG	FUPG	p
N		23	16	13	
Age (year)		44.3±10.7	43.1±12.6	39±14.5	>0.05
Gender	Male Female	10 (43.5%) 13 (56.5%)	6 (37.5%) 10 (62.5%)	4 (30.8%) 9 (69.2%)	>0.05
BMI		21.1±2.0	19.6±2.1	20.6±2.6	>0.05

NDG: New diagnosed Graves patients (The BMI of this group was calculated at month 0), FUPG: Follow-up Graves patients, BMI: Body mass index.

We measured the levels of free triiodothyronine (T3), free thyroxine (T4), thyroglobulin, and thyroid-stimulating hormone (TSH) in the blood samples of the participants. There is a significant increase in the free T3 and T4 levels of the blood samples of the NDG group compared to that of healthy individuals. Furthermore, the blood TSH levels of NDG and FUPG groups were found significantly diminished compared to healthy individuals. It was indicated that the decreased TSH levels accompanying enhanced free T3 and T4 levels in blood samples of the participants in the NDG and FUPG groups compared to control confirmed that the participants in the NDG and FUPG groups had uncontrolled hyperthyroidism (Table 2). TSH receptor antibody, anti-TPO, and anti-thyroglobulin blood levels of the participants in the NDG and FUPG groups were found to be greater than that of the control group, as detailed in Table 2.

Table 2. The thyroid function tests, thyroid autoantibody levels, biochemical data, and hemogram findings of the participants. Data were presented as mean±SEM. *p<0,05, compared to control

Thyroid tests	Control	NDG		FUPG
		0th month	3rd Month	
N	23	16		13
TSH (0.27-4.2 IU/ml)	1.6±0.6	0.0*	0.9±1.0*	0.1±0.3*
T3 (2.3-4.5 pg/ml)	3.3±0.7	12.3±9.8*	5.4±3.5*	4.0±1.2*
T4 (0.93-1.7 ng/dl)	1.2±0.4	2.4±1.6*	1.5±0.8*	1.8±0.8*
TSH receptor antibody (0-1,5 U/L)	0.3±0.2	6.8±4.6*	5.3±6.0*	7.3±6.1*
Anti-TPO (0-34 IU/ml)	7.0±10.2	185.1±157.5*	-	84±92.9*
Anti-thyroglobulin (0-115 IU/ml)	38.1±104.1	132.4±129.7*	-	90.7±126.5*
Thyroglobulin (3,5-77 ng/ml)	23.5±20.7	73.9±59.6	-	107.9±50
Blood data of the participants	**Control**	**NDG (0th month)**		**FUPG**
Calcium (8,6-10,2 mg/dl)	9.2±0.4	9.7±0.4+		9.6±0.40
Albumin (3,5-5,2 g/dl)	4.6±0.3	4.3±0.2+		4.4±0.4
Phosphorus (2,7-4,5 mg/dl)	3.5±0.5	3.9±0.9+		3.9±1.0
Alkaline phosphatase (0-104 U/L)	69.6±32.4	97.6±24.2*+		70.0±31.7
AST (0-31 U/L)	17.1±3.5	21.1±10.1+		21.4±9.7
ALT (0-33 U/L)	21±13.3	24.7±22.7+		25.0±18.0
Hemoglobin (g/dl)	14.3±1.6	13.6±1+		13.6±1.6
Leukocyte (10^3/ul)	7.2±2.1	7.0±1.9+		6.9±2.6
Neutrophil (10^3/ul)	4.1±1.6	3.6±1.0+		4.0±1.6
Lymphocyte (10^3/ul)	2.3±0.8	2.7±1.1+		2.6±1.1
Platelets (10^3/ul)	269.1±60.4	288.1±45.6+		272.6±71.6

TSH: Thyroid stimulating hormone, T3: Free Triiodothyronine, T4: Free thyroxine, TSH Receptor Antibody: Anti-thyroid stimulating hormone receptor antibodies, Anti-TPO: Anti-thyroid peroxidase antibodies, AST: Aspartate transaminase, ALT: Alanine transaminase. + (The data of this group was obtained at month 0)

The serum NPT levels of the participants in the NDG and FUPG groups were considerably higher than the control group, as listed in Table 3 and Figure 1. Although the groups were similar in terms of age, gender, and BMI, when neopterin was compared between the groups, it was determined that age, gender, and BMI were not confounding factors (p>0.05). ESR and CRP levels, which are commonly used to evaluate inflammation, were found to be similar between the patient and control groups. In contrast, neopterin levels were found to differ significantly between the groups, being higher in the patient groups.

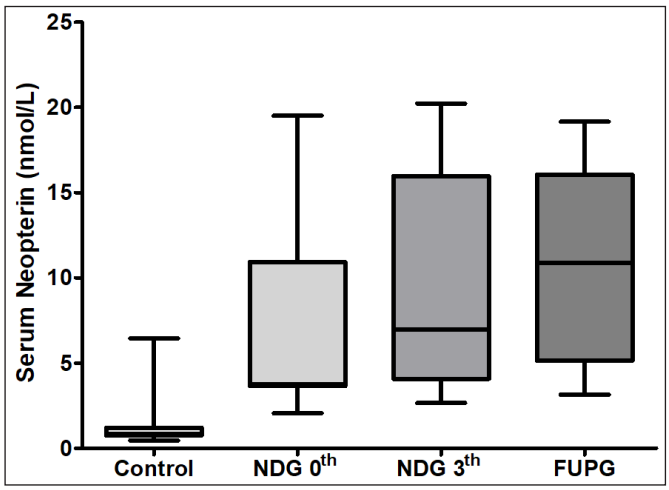

Figure 1. Serum NPT of participants
NDG: New diagnosed Graves patients (0th month, 3rd month), FUPG: Follow-up Graves patients.

Table 3. NPT, ESR, and CRP levels of participants. Data were presented as mean±SEM. *p<0,05, compared to control

		N	Serum neopterin (nmol/L)	ESR (0-10 mm/h)	CRP (0-5 mg/L)
Control		23	1.44±1.45	9.8±4.9	1.3±1.0
NDG	0th month	16	6.66±5.28*	11.7±6.7	1.9±1.3
	3rd month	16	9.24±6.12*	10.6±4.2	1.8±1.3
FUPG		13	10.68±5.63*	12.2±5.5	2.0±1.2

NPT: Neopterin, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein.

Despite the high TRAb values in the NDG and FUPG groups, we could not detect a relationship between TRAb values and neopterin levels (r=-0.270, p:0.377).

DISCUSSION

NPT, considered a biomarker, is attracting widespread interest in the diagnosis of infectious inflammatory diseases and malignancies. Immune activation stimulates T-lymphocytes to secrete cytokines like IL-2 and IFN-γ. NPT is produced in macrophages activated with IFN-γ secreted from T-lymphocytes in inflammatory conditions. Increased generation of reactive oxidative substances in inflammatory conditions enhances the production of NPT obtained from BH_4, which plays an important role in defense mechanisms against oxidative stress.[7,8]

Various studies indicate that serum NPT levels are consistent with the severity and progression of infectious and inflammatory diseases and malignancies. The highest

serum NPT levels were determined in the acute phase of inflammatory diseases such as rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis, autoimmune thyroiditis, systemic lupus erythematosus, and early-onset autoimmune diabetes. Also, it was established that NPT levels in biological fluids changed with disease severity and expansion.[8,9,11] Another previous research established that the NPT levels in synovial fluid samples of RA patients were greater than in control groups. A considerable increase in NPT levels in synovial fluid samples of RA patients was detected compared to those with osteoarthritis. The relative increase in NPT levels in synovial fluid samples of RA patients accounted for cellular stimulation of immune reaction.[12] In contrast, serum NPT levels were found to be higher in serum samples of the patients with systemic lupus erythematosus (SLE) compared to the control. On the other hand, a marked increase was determined in synovial fluid samples of RA patients compared to healthy individuals in the same study.[13] Therefore, previous research conducted with Arshadi et al.[14] indicated that the disease activity determined with the DAS-CRP method in the patients with RA demonstrated a correlation with the plasma NPT levels compared to healthy individuals. The evidence points to the likelihood that NPT is a sensitive marker for the activity of inflammatory diseases arising from macrophage activation induced by T-lymphocytes.

The results obtained from this study indicated that serum NPT levels of the patients with Graves disease displayed a marked increase compared to healthy individuals. Also, it was determined that the serum NPT level of the patients with followed-up Graves disease (FUPG group) was found to be higher than that of the patients who have newly diagnosed Graves disease (NDG group). These results are unlike the evidence in the literature, which suggested that serum NPT levels were decreased with treatment.[15,16] The marked increase in serum NPT levels of the followed-up Graves disease patients may be attributed to enhanced disease activity by the cellular immune response against thyroidal tissue. Neopterin is released from macrophages with the stimulation of IFN-γ released from T helper cells. Major histocompatibility antigen (MHC) class II, which provides antigen presentation to T helper cells in healthy individuals, is expressed only on antigen-presenting cells (APCs), not thyroid cells. It is thought that thyroid cells also express MHC class II molecules (notably HLA-DR molecules) with the stimulation of interferon released by any thyroidal T cell in Graves' patients, exacerbating the already established thyroid autoimmunity.[17]

In a previous study conducted by Wagner et al.[9] serum NPT levels, considered a T-lymphocyte/monocyte axis function biomarker, were determined to be greater in the patients with Graves disease or autoimmune thyroiditis compared to healthy individuals and the patients with nontoxic goiter. However, contrary to our results obtained from this study, no significant difference in serum NPT levels was established between the different stages of Graves disease, such as newly diagnosed, treated, remission, and relapse. Although it has been suggested that the NPT levels may increase due to increased thyroid volume and macrophage numbers in Graves disease, no correlation was found between them in that study. Similarly, no relationship was found between NPT and thyroid volume in Schomburg et al.'s study.[18]

Zantut-Wittmann et al.'s[19] study showed ATDs reduced HLA-DR expression in follicular cells in fine-needle aspiration biopsy samples of Graves patients with controlled thyrotoxicosis. Therefore, NPT levels associated with HLA-DR expression can be expected to decrease as the duration of ATD treatment increases. In our study, while NDG 0 months had not yet received ATD, NDG 3 months and FUPG patients received ATD but were not yet in a euthyroid state. Since we worked with patients with uncontrolled thyrotoxicosis, we may have concluded that NPT levels are higher with longer disease duration and that ATD treatment does not reduce NPT. In Schomburg's study[18], no significant difference was found in NPT levels between Graves patients who received and did not receive ATD. Although our study lacks a group that did not receive ATD, we can say that ATD treatment does not significantly affect NPT levels in patients who have not yet reached remission.

The TRAb, anti-TPO, and anti-thyroglobulin antibody levels of the NDG and FUPG groups participants were higher than the control group in our study. Since only the patients in the active phase of the disease were included in the study, and the disease control was not complete in the FUPG group, TRAb values were very similar to those in the NDG group. NPT levels in biological fluids are altered with T-lymphocyte/monocyte activation in autoimmune reactions. Considering the high NPT levels, our study showed that the antibody formation against thyroidal proteins like TSH-receptor, TPO, and thyroglobulin in patients with Graves disease results from the activation of CD4+ T-helper lymphocytes. In recent research by our study group, NPT levels were found to be lower in patients with subacute thyroiditis than in controls. It was inferred that CD8+ cytotoxic T-lymphocytes in the thyroidal tissue have a pivotal role in the immune reaction in subacute thyroiditis rather than CD4+ T-helper cells. Also, it was assumed that lower serum NPT levels in the patients with subacute thyroiditis were associated with the involvement of HLA-B*35- MHC class I in the pathogenesis of subacute thyroiditis.[11,20]

Neopterin shows the extent of inflammation related to cellular immunity and can sometimes result differently from the general inflammatory markers ESR and CRP. In our study, there was no difference in ESR and CRP

between the patient and the control groups and intergroup comparisons of the patients. Still, the difference in NPT levels reveals that NPT is a unique inflammatory marker.

The present descriptive research has some limitations that the sample size was not sufficient. Our results are promising and should be verified in a larger cohort of patients with Graves disease, including those in remission and those with relapses. Further investigations should focus on the local NPT production in the thyroidal tissue.

CONCLUSION

It is conceivable that monitoring NPT levels in biological fluids could provide compelling evidence in evaluating the diagnosis, prognosis, and treatment efficacy of autoimmune diseases. Also, NPT might be put forward as a potential biomarker to determine the diagnosis, disease stage, treatment efficacy, and immunological remission status of Graves disease.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Eskisehir Osmangazi University Non-interventional Clinical Researches Ethics Committee (Date: 03.11.2020, Decision No: 2020-10).

Informed Consent: Written informed consent form was obtained from patients.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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